Similar shells are not necessarily a reliable guide to phylogeny: *Rissoa guerinii* Récluz, 1843, and *Rissoa lia* (Monterosato, 1884) (Caenogastropoda: Rissoidae): a case study

Francesco Criscione[1]
Francesco Paolo Patti[2]
Functional and Evolutionary Ecology Laboratory
Stazione Zoologica "Anton Dohrn"
P.ta S. Pietro, 1, 80077
Ischia (NA), ITALY

ABSTRACT

According to the recent theory of speciation through loss of planktotrophy, the pairs of northeastern Atlantic species of caenogastropods would be the result of a cladogenetic event in which a planktotrophic ancestor gave rise to two geographically separated species with different modes of larval development, but retained virtually identical teleoconch characters. This idea was proposed as the working hypothesis for the origin of the pair of supposed sibling species *Rissoa guerinii* Récluz, 1843 and *R. lia* (Monterosato, 1884). The present study shows that claims of a close morphological resemblance between the two species are unjustified as a considerable divergence emerged in shell geometric morphometric analysis and in 16S rRNA mitochondrial gene. We conclude that *R. guerinii* and *R. lia* cannot be regarded as sister or cryptic species and should no longer be considered a planktotrophic/non-planktotrophic pair.

Additional keywords: Gastropoda, sibling species, larval development strategy, mitochondrial DNA, geometric morphometry

INTRODUCTION

Many authors (e.g. Jablonski and Lutz, 1983) identified two main categories of developmental strategies in marine invertebrates: planktrotrophy (P), with larvae feeding on plankton, and non-planktotrophy (NP), with planktonic larvae feeding only on their yolk supply (lecithotrophy), or with direct development.

According to the so-called "shell-apex rule" (Thorson, 1950), larval development in marine gastropods can be inferred from observations of the protoconch. A multispiral protoconch and a smaller dimension of the initial whorl accounts for a planktotrophic developmental mode, whereas a larger paucispiral protoconch is directly linked to non-planktotrophy.

There are several examples of northeastern Atlantic caenogastropod pairs of related taxa that possess identical teleoconchs and differ exclusively in protoconch characters. Oliverio (1996) provided a list of 28 of these P/NP pairs and indicated that their specific status is still a matter of debate.

Verduin (1986) maintained that in species of *Rissoa* (Rissoidae) the occurrence of this phenomenon is of remarkable extent and nine pairs of Oliverio's list were species of this genus. In his revision of the genus, Verduin (1976, 1982, 1985, 1986) considered differences in developmental mode to definitively separate species, thus regarding each member of a P/NP pair as a distinct species. His opinion was commonly accepted (e.g. Bouchet, 1989), although some authors have more recently suggested the possibility of intraspecific variability in larval development (Warén, 1996; Cadée, 1998) or that this variation indicated incipient speciation (Rehfeldt, 1968; Panico and Patti, 2005).

Oliverio (1996) suggested a mechanism of speciation explaining the origin of P/NP pairs in the NE Atlantic region. This involved the modification of larval development, with one species abandoning planktotrophic feeding, thus giving rise to another species. From the observation that in the Mediterranean the non-planktotrophic mode is more dominant in the Eastern basin, Oliverio (1996) suggested a paleogeographic model to explain the reasons of speciation through the loss of planktotrophy. During glacial periods sea level lows caused isolation between the Mediterranean and the Atlantic, and between the Eastern and Western Mediterranean basins. The resulting conditions gave rise to factors thought to select against planktotrophic larvae (fluctuations in the energy input, restricted areas, and higher predation pressure; Strathmann, 1978a, b). These factors may have caused the shift from planktotrophy to lecitotrophy resulting in speciation and origin of a P/NP pair.

[1] Current address: Malacology Section, Australian Museum, 6 College Street, 2010 Sydney, NSW Australia. francesco.criscione@austmus.gov.au
[2] fpatti@szn.it; both authors are corresponding authors

Within this scenario, species forming a P/NP pair can be considered sibling species (sensu Knowlton, 1986), being both cryptic (i.e., difficult to distinguish using the traditional morphological characters) and sister (i.e., sharing the same ancestor).

After the examination of a large amount of museum material of *Rissoa guerinii* Récluz, 1843, and *Rissoa lia* (Monterosato), 1884 (Rissoidae), Verduin (1985) highlighted a strong resemblance in shell characters between these species and concluded that they "may often only be identified reliably by their type of apex" (Verduin 1986: 14). Their distribution (Verduin, 1985) overlaps in the Western Mediterranean, but only *R. guerinii*, occurs in the Atlantic and is absent from the Adriatic and Aegean, where *R. lia* is present.

In this work we provide, based on both dry and live-collected material, a critical reinterpretation of the two taxa and investigate their phylogenetic relationships.

MATERIALS AND METHODS

The source of the biological material examined in this study is twofold. Snails from field sampling and empty shells belonging to the historical collection of Philippe Dautzenberg (housed in the Royal Belgian Institute of Natural History (RBINS), Brussels) have been used in this research.

Live-collected Material

Sampling of living material was performed in the infralittoral of Santa Tecla, Sicily (Mediterranean, Ionian Sea), where both species commonly occur (Scuderi, pers. comm.), at 1–5 m depth in Dec. 2005, Apr., Jun., and Nov. 2006. About 0.03 m^3 of the red alga *Pterocladiella capillacea* (S. G. Gmelin) Santelices and Hommersand was collected by SCUBA diving for each sample. Material was immersed in seawater and transferred to the laboratory. Each sample was divided into 20 subsamples that were individually washed in a tank containing 5 l of 50% seawater for not more than five minutes. Osmotic shock provided caused the vagile fauna to detach from the algal thallus and fall in the bottom of the tank, from where the specimens were quickly collected and placed in seawater. Living material was sorted and taxonomically determined under a Wild Makroskop M420 stereoscopic microscope. Specimens belonging to *Rissoa guerinii* and *R. lia* were isolated; their sex was determined by checking the presence of a penis in the right side of pallial cavity. Some were placed separately in running seawater at 18°C and provided with fresh *P. capillacea* thalli, others were immediately preserved in 80% ethanol. Reared specimens did not survive more than 6 weeks; empty shells of dead specimens were retained and used for observation.

Shells and Head-foot: Adult living specimens of both *R. guerinii* and *R. lia* were placed in a Petri dish with seawater. Shells were held with forceps and the snails attempted to crawl extending their foot completely, enabling the head-foot to be observed in detail. Images were taken and digitized using a Leica DFC 300 FX video camera and Leica Application Suite version 2.4.0 software. Color drawings were also made to better represent the color pattern of the head-foot.

Traditional Shell Morphometry: Standard teleoconch (L, M, W_{N-1}, D_{N-1}) and protoconch parameters (d and D_0) (Verduin, 1982a) were measured on a sample of 100 randomly selected shells (from 53 males and 47 females) from live-collected material of each species. The total number of shell whorls (N) was also counted. The following standard teleoconch ratios were calculated: relative height (L/N), slenderness (L/D_{N-1}), relative aperture height (M /L) and last-whorl height over width ratio (W_{N-1}/ D_{N-1}).

A principal component analysis (PCA) and a discriminant analysis (DA) were performed on the dataset obtained combining protoconch parameters and teleoconch ratios. Tests and plots were implemented by SPSS v.15 statistical software (© SPSS Inc., 2006) and by SYSTAT statistic software v. 12 (Wilkinson et al., 1992).

Geometric Shell Morphometry: Fifteen shells (8 females and 7 males) at terminal growth were randomly selected from live-collected material of each species. Shells were observed using a Leica Z16 APO stereoscopic microscope, and colour images were taken and digitized using a Leica DFC 300 FX video camera and Leica Application Suite version 2.4.0 software. The shells were always placed in the same position, with the coiling axis in vertical position and the aperture on the same plane as the objective (Carvájal-Rodríguez et al., 2005). Using the software tpsDIG2 v. 2.10 (Rohlf, 2007a), 19 landmarks (LM) were established (Figure 1). LM1 is the apex of the shell; LM2, LM4 and LM6 are placed on the right border of the profile at the beginning of the three last complete whorls. LM15, LM17, and LM19 are the corresponding landmarks on the left border of the profile. LM3, LM5, LM16, and LM18 mark the intermediate position respectively between LM2 and LM4, LM4 and LM6, LM15 and LM17, LM17, and LM19 along the curvature of the whorl; LM8 is at the lower suture of the last complete whorl and LM7 marks the intermediate position between LM6 and LM8 along the curvature of the whorl. LM9 is the most external position in the upper part of the outer lip; LM10 and LM12 are the most external positions respectively in the external right and left part of the outer lip; LM11 is the lowest point at the base; LM14 is the most external point in the last whorl at the left profile of the shell; LM13 is the profile point between LM12 and LM14 (closest to LM7). As described in Carvájal-Rodríguez et al. (2005) the matrix of raw coordinates generated by tpsDIG2 was used in tpsRelw v.1.45 (Rohlf, 2007b) to compute shell size (CS), uniform (U1 and U2) and non-uniform (several relative warps, RWs) shape components for each specimen. Standard

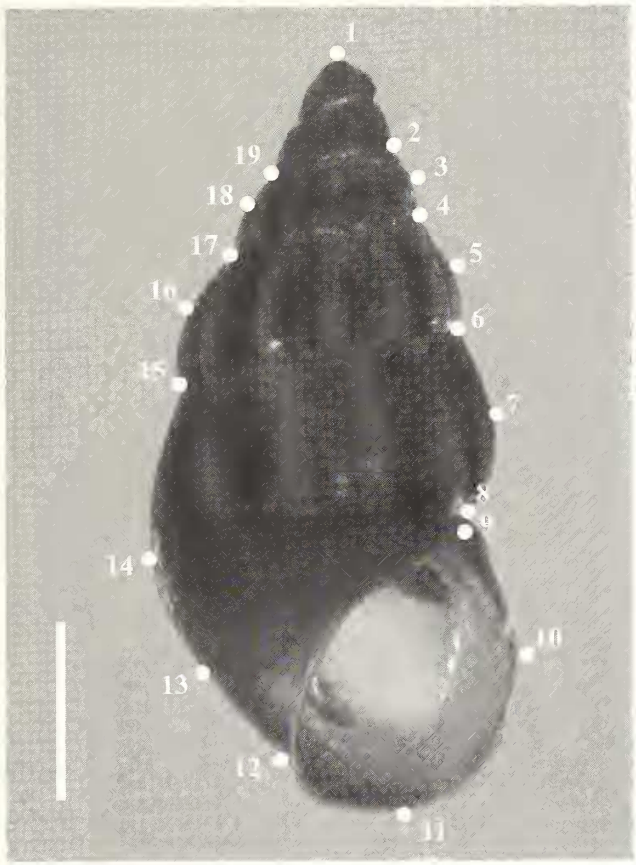


Figure 1. Shell of a live-collected *R. lia* from S. Tecla showing the placement of the 19 landmarks used for geometric morphometric analysis. Scale bar = 1 mm.

parametric tests were performed on the obtained variables using the SPSS/PC package v. 15.0.

Radular Analysis: The shell of 10 live-collected specimens of each species was removed and the bodies incubated for 3–4 hours at 50°C in 70% KOH solution. After complete tissue dissolution, radulae were isolated, rinsed in distilled water, and mounted on SEM stubs. Observation and pictures were made using a Jeol JSM-6700F scanning electron microscope. The cusps of rachidian and lateral teeth were counted and frequency histograms were drawn to show differences in cusps arrangement between the two species.

Molecular Systematics: Forty-five ethanol preserved specimens of *R. guerinii* and 34 of *R. lia* were randomly selected from live-collected material. The shell of each specimen was broken in a mortar and removed. DNA extraction plus amplification, purification and sequencing of a 337 bp segment of mitochondrial 16S rRNA gene were performed as described in Criscione et al., 2009.

Sequences obtained were aligned with CodonCode Aligner v. 1.6.3 (CodonCode Corporation, Dedham, MA) and the alignment refined by eye. For all samples, both forward and reverse strands were analysed. Genbank accession numbers for sequences used in the analysis are given in Table 2.

For the sequences generated, Parsimony and Maximum likelihood trees were obtained using PAUP* v. 4.04 (Swofford, 2003). The program Modeltest version 3.06 (Posada and Crandall, 1998) was employed to select HKY+I model in Maximum Likelihood. In computing trees, the option of 1000 bootstrap replicates was selected. A reduced median joining network (MJ) (Bandelt et al., 1999) was obtained with the software Network v. 4.5 (http://www.fluxus-technology.com/NETW4500.exe).

Table 1. List of studied material in Dautzenberg collection (RBINS).

Acronym	No. of shells contained	Main original label
Rga	About 80 *R. guerinii*	*Rissoa guerini* Recl. var. *albina* Dautz./Rochebonne/dd. 16.IX.05
Rgb	About 60 *R. guerinii*	*Rissoa guerini* Recl. var. *bipartita* Dz and Dur./Rochebonne/dd. 16.IX.05
Rgc	About 80 *R. guerinii*	*Rissoa guerini* Recl. var. *conspersa* Dautz. and Durouchoux/ Rochebonne/dd. 16-IX.05
Rlma	Many *R. lia*	*R. (Apicularia)* lia, Benoit/typique de Messina!/Monterosato 2.III.17
Rlmb	5 *R. lia*	bis/R. (*Apicularia*) lia, Benoit/ Messina! Playa
Rlp	8 *R. lia*	*Rissoa lia* Monts./Paulille/Bucquoy
Rlt	many *R. lia*	*Rissoa lia* Benoit/Tanger/Pallary 1. 7. 98

Table 2. GenBank accession numbers for the sequences used in the analysis. Accession numbers with the prefix GU were collected for this study.

Taxon	AN	Analysis
R. guerinii	from GU177882 to GU177921	NJ, MP, ML, MJ
R. lia	from GU177922 to GU177955	NJ, MP, ML, MJ
R. similis	GU177963	NJ, MP, ML, MJ
R. labiosa	AY676117	NJ, MP, ML, MJ
R. parva	AF445343	NJ, MP, ML, MJ
R. auriscalpium	GU177879	MJ
R. variabilis	GU177880	MJ
R. violacea	GU177881	MJ

Dautzenberg Collection Material

The relevant material in the Dautzenberg collection was examined using an Olympus® SZX10 stereoscopic microscope. After this survey, 15 lots were selected as on their historical value as vouchers (Verduin, 1985) and the specific determination of the shells contained

was verified according to current taxonomy. Clearly misclassified specimens in the lots were temporarily removed and not further considered. Several specimens of each lot were observed using the stereomicroscope and digital pictures were taken with an Olympus® CAMEDIA C-7070 WZ digital camera. Table 1 contains the list of the lots studied, accompanied by the data on the original label, the number of specimens present and photographed, and their revised species determination. An acronym is given to enable identification of the lot in the following text.

RESULTS

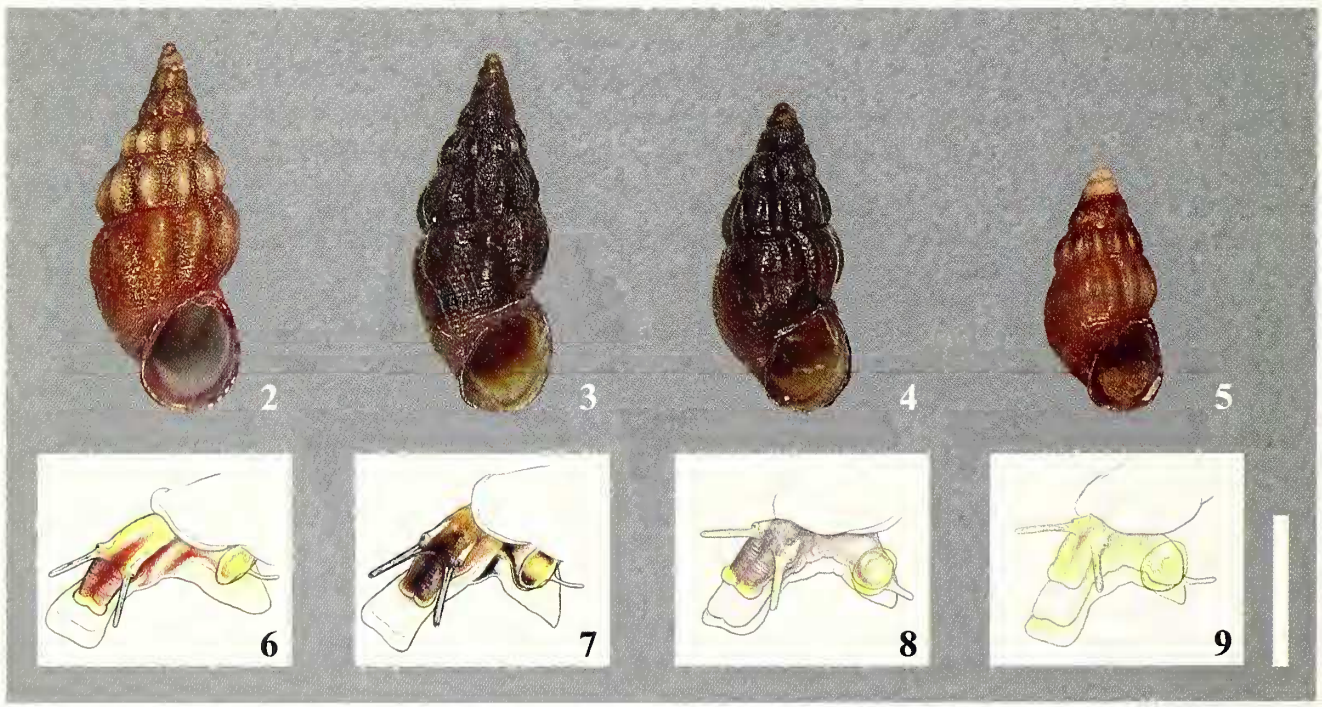
Live-collected Material

***Rissoa guerinii* Shell:** Shell spindle-shaped; apex solid, sharp and glossy; whorls 7, apical 2–3 nearly flat-sided, 3–4 youngest ribbed and more convex, with penultimate whorl generally bulging out and giving shell characteristic fusiform shape. Shells of females always bigger than those of males with same number of whorls.

Shell ornamentation composed by axial ribs and intervening furrows, spiral ridges and intervening grooves, and growth lines. Shells devoid of spiral ornamentation rarely found. Axial ribs limited to youngest 3–4 whorls, 11–14 per whorl, robust and prominent, swelling in middle of whorl and fading toward shell base; normally slightly opisthocline, tending to prosocline on body whorl near aperture and close to suture being slightly flexuous; each rib equal in breadth to interspaces. Extremely weak labral rib, often having appearance of whitish smudge as broad as two axial ribs, starting from end of last axial rib and ending before outer lip. Spiral ridges with grooves delicately and closely cancellated as a result of intersection with growth lines. Growth lines prosocline and running obliquely over ribs and furrows. Aperture as described by Fretter and Graham (1978). Two distinct shell colour varieties detected: typical and "var. *conspersa*" (Dautzenberg and Durouchoux, 1914). Typical *R. guerinii* (Figure 2) with ribless white-brownish or lilac whorls, youngest showing sinuous brown spiral lines corresponding to furrows of ribbed whorls. Ribless whorls with fawn or brown background colour on furrows among ribs and on shell base (sometimes tinged pale lilac up to base of ribs). A checkerboard pattern (or a series of zigzag lines) created by background color and a whitish color occasionally present. Ribs always whitish or pale lilac. Peristome pale violet, throat with brown-lilac band extending on to columella.

Rissoa guerinii "var. *conspersa*" (Figure 3) with entire shell covered by a uniform chess board pattern or a series of zigzag lines (as described for some typical shells), with predominance of a dark brown colour over whitish colour. Ribs often encircled by a white line, normally interrupted in correspondence of intervening furrows. Apex, peristome, and inner part of aperture as described for typical pattern.

***Rissoa guerinii* Head-foot:** As for shells, two colour types detected (Figure 6, 7), viz. typical *R. guerinii* and *R. guerinii* "var. *conspersa*" (Dautzenberg and



Figures 2–9. Pictures of shells and drawings of soft body parts of *R. guerinii* typical (2, 6), *R. guerinii* "var. *conspersa*" (3, 7), *R. lia* "var. *castanea*" (4, 8) and *R. lia* "var. *fulva*" (5, 9). Scale bar = 1 mm. Drawings by D. Scuderi.

Durouchoux, 1914). Foot always whitish and median part of sole stained brown, lighter in typical *R. gnerinii* than in "var. *conspersa*." Snout light brown in *R. gnerinii* typical and darker brown in "var. *conspersa*." Margins of distal portion of snout and remaining part of head yellowish in *R. gnerinii* and light brown in "var. *conspersa*." Cephalic tentacles whitish, but sometimes dark brown in "var. *conspersa*." A whitish spot behind base of cephalic tentacles always present.

***Rissoa lia* Shell:** Shell conical, apex solid, obtuse and opaque; whorls 6, all equal-sided and tumid, apical 2 whorls ribless, others ribbed or ribless (though last whorl often ribless), penultimate generally not bulging out rest. Sexual dimorphism as for *R. gnerinii*. Shell ornamentation of axial ribs and intervening furrows, spiral ridges and intervening grooves, and growth lines. No shells devoid of spiral ornamentation found. Axial ribs (when present) always 14 per whorl, strong and prominent, with same thickness across whorl but fading towards base on body whorl; normally slightly opisthocline, tending to be prosocline; each rib slightly narrower than intervening furrow. No labral rib or whitish smudge before peristome present. Spiral ornamentation as for *R. gnerinii*. Aperture oval or D-shaped, peristome not showing sinuses or slight projection of inner lip; edge very thin, beveled internally turning out to form a though thin flange. Outer lip arising below periphery of body whorl, (somewhat below level at which ribs end), its curvature initially slight or sometimes nearly straight. Columella short, peristome everted over a groove, no umbilicus present. Two distinct color varieties detected, here named *castanea* and *fulva* (after Monterosato, 1884). Intermediate specimens rarely found. *R lia* "var. *castanea*" (Figure 4) violet-brownish to dark brown with ribs always lighter. First two whorls white but sometimes brown to dark brown. Peristome violet-brownish. *R. lia* "var. *fulva*" (Figure 5) uniformly fawn with ribs always lighter or even whitish. First two whorls fawn or white. Peristome as in "var. *castanea*", but neck with a brown-lilac band running from lower border of labral rib to columella.

***Rissoa lia* Head-foot:** As for *R. gnerinii*, two different color types detected remarkably distinct in colour pattern and corresponding to shell colour variety and thus named *castanea* and *fulva* (Figures 8, 9). Foot always entirely whitish and cephalic tentacles always yellowish with a whitish spot behind their base. In *R. lia* "var. *castanea*" snout dark and its distal part yellowish, in "var. *fulva*" snout yellowish, often with a short brownish stripe running along median part.

Traditional Morphometry: Among the 6 principal components extracted, PC1 (55%) and PC2 (26%) explained most of the variance observed (Table 3). Component matrix (Table 4) and loading plot (Figure 10) illustrate the correlation between principal components and shell variables. PC1 is a strongly positively correlated with L/N, L/D_{N-1} and W_{N-1}/ D_{N-1}, strongly negatively correlated with M/L and weakly negatively correlated with d and D_0. PC2 is not significantly correlated with teleoconch ratios but it is strongly positively correlated with d and D_0.

No clear clusters resulted from plotting PC1 value of each shell against its respective PC2 value (Figure 11). The 95% confidence ellipse of *R. gnerinii* contains almost only positive values of PC1, but both positive and negative values of PC2 whereas that of *R. lia* encircles mostly negative values of both PC1 and PC2.

The eigenvalue of discriminant function between the two species was 1.307, the canonical correlation 0.753 and the Wilks' lambda (0.433) was highly significant ($p<0.001$). The structure matrix (Table 5) shows the cumulative within-groups correlations between discriminating shell variables and the standardized canonical discriminant function obtained. Table 6 shows the results of the classification statistics obtained using the values of the discriminant function of each individual to predict its *a posteriori* species membership.

Geometric Morphometry: Table 8 shows the percentages and a descriptive statistical summary of the

Table 4. Component matrix for the first two principal components.

	Component	
	1	2
D	–0.271	0.864
D_0	–0.318	0.848
L/N	0.843	0.181
L/D_{N-1}	0.914	0.134
M/L	–0.913	–0.151
W_{N-1}/ D_{N-1}	0.860	0.106

Table 3. Total variance explained by single principal components.

	Initial Eigenvalues			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.294	54.901	54.9	3.294	54.901	54.9
2	1.551	25.852	80.8	1.551	25.852	80.8
3	0.432	7.193	87.9			
4	0.342	5.704	93.6			
5	0.235	3.909	97.6			
6	0.147	2.442	100.0			

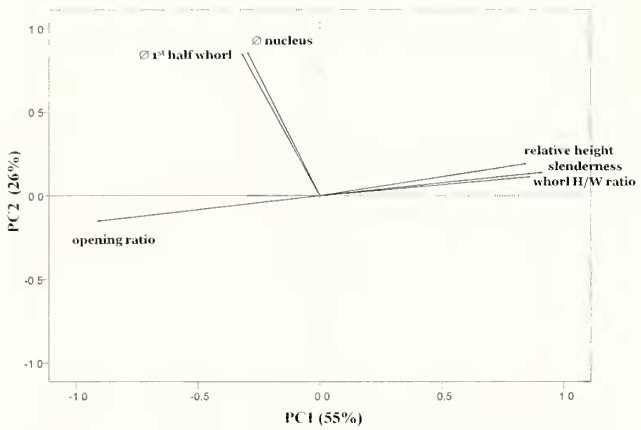


Figure 10. Loading plot of the shell variables relative to the first two principal components.

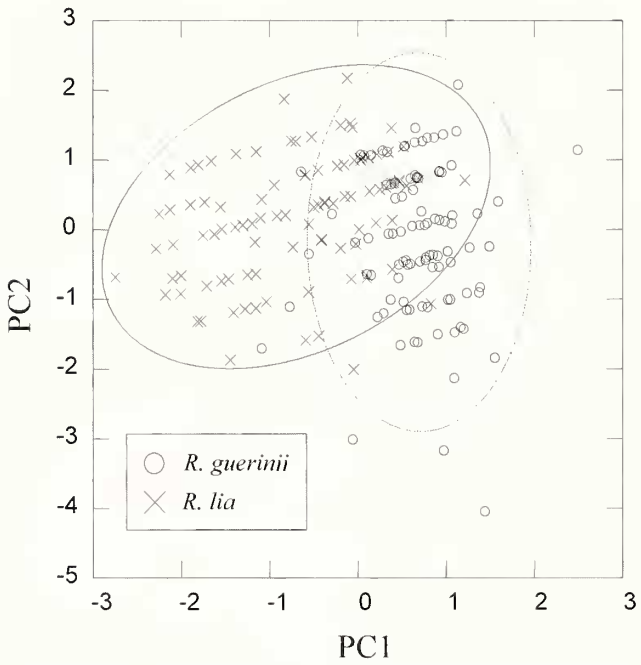


Figure 11. Scatterplot of the first two principal components. 95% confidence ellipses are drawn for each species. Dashed ellipse represents *R. guerinii*.

Table 5. Structure matrix showing the loadings of each shell variable on the discriminant function. The variables are ordered by absolute size of correlation within discriminant function.

	Discriminant Function
M/L	0.717
L/N	–0.699
L/D_{N-1}	–0.672
W_{N-1}/ D_{N-1}	-0.487
d	0.382
D_0	0.307

Table 6. Classification summary for the discriminant analysis.

Species		*R. guerinii*	*R. lia*	Total
Count	*R. guerinii*	93	7	100
	R. lia	14	86	100
%	*R. guerinii*	93.0	7.0	100.0
	R. lia	14.0	86.0	100.0

Table 7. Multiple regression model testing allometry for the non-uniform shell shape variables.

Multiple regression		Variables in the model	
r^2	F	Name	Beta
0.5	20.3^{***}	RW2	-0.648^{***}
		RW9	0.282^{**}

$^{**}P < 0.05$, $^{***}P < 0.001$.

relative score for CS, the two uniform components and the first 10 RWs, explaining more than the 91% of the overall variation. Table 7 shows the results of the allometric analysis for shell shape measurements conducted by step-wise multiple regression analysis for centroid size (as dependent variable) and two uniform and 29 non-uniform measurements, as independent variables. The F test of the regression analysis was highly significant ($p<0.001$) and two of the relative warps, RW2 and RW9, contributed significantly to the regression model on the centroid size. Centroid size, uniform components and only the first ten relative warps were considered in the analysis of variance (ANOVA), performed to evaluate the significance of differences in size and shape variables. Shells of *R. guerinii* and *R. lia* differed significantly in CS ($p<0.001$), RW1 ($p<0.05$) and RW2 ($p<0.05$). The cumulative results of the analysis are shown in Table 8. The significance level obtained for the corrected analysis (ANCOVA) with centroid size as covariate was not maintained for the difference in RW1, but was not affected for RW2. The eigenvalue of the stepwise discriminant function between the two species, calculated for the 29 RWs, was 3.199, the canonical correlation 0.873 and Wilks' lambda (0.238) was highly significant ($p<0.001$), indicating a good separation between groups. Twelve shape variables contributed to the discriminant function (RW1, RW2, RW4, RW5, RW6, RW7, RW9, RW10, RW18, RW23, and RW27). The standardized coefficient matrix (Table 9) shows the relative importance of the independent variables in determining the standardized canonical discriminant function. Using the individual values of the discriminant functions to predict *a posteriori* species memberships, 26 (86.7%) individuals out of 30, were assigned to the correct species, leaving only 4 (13.3%) that were erroneously classified. Looking at species statistics (Table 10), 13 (86.7%) specimens of *R. guerinii* were correctly assigned to this species and only two (13.3%) were assigned to *R. lia*. The same percentages of correctly/

Table 8. Descriptive statistical summary and results of ANOVA and ANCOVA for the main shell size and shape variables between *R. guerinii* and *R. lia*. **P < 0.05, *** P < 0.001, ns = non significant.

Measure		CS	U1	U2	RW1	RW2	RW3	RW4	RW5	RW6	RW7	RW8	RW9	RW10
Variance explained					32.2%	19.4%	14.5%	8.2%	4.2%	3.6%	3.2%	2.5%	2.0%	1.9%
R. guerinii	Mean	1020	–0.001	0.001	0.012	–0.011	–0.002	–0.004	–0.003	0.003	–0.003	0.000	0.002	–0.001
	SD	122	0.014	0.010	0.038	0.016	0.025	0.018	0.012	0.010	0.010	0.010	0.009	0.008
R. lia	Mean	815	0.001	–0.001	–0.012	0.011	0.002	0.004	0.003	–0.003	0.003	0.000	–0.002	0.001
	SD	128	0.012	0.009	0.016	0.026	0.017	0.012	0.010	0.010	0.009	0.008	0.006	0.007
ANOVA		20.10***	0.11 ns	0.45 ns	4.79**	7.97**	0.32 ns	2.05 ns	2.42 ns	1.96 ns	2.16 ns	0.06 ns	3.43 ns	0.75 ns
ANCOVA			1.06 ns	0.46 ns	3.21 ns	9.96**	0.20 ns	1.00 ns	1.38 ns	1.19 ns	1.57 ns	0.15 ns	1.79 ns	2.01 ns

Table 9. Standardized coefficient matrix showing the relative importance of the shape variables.

	Discriminant function
RW2	1.501
RW1	–1.276
RW9	–1.127
RW5	0.978
RW7	0.932
RW4	0.911
RW6	–0.893
RW23	–0.789
RW27	0.716
RW18	–0.685

Table 10. Summary of classification results obtained employing the discriminant function.

Species		*R. guerinii*	*R. lia*	Total
Count	*R. guerinii*	13	2	15
	R. lia	2	13	15
%	*R. guerinii*	86.7	13.3	100.0
	R. lia	86.7	13.3	100.0

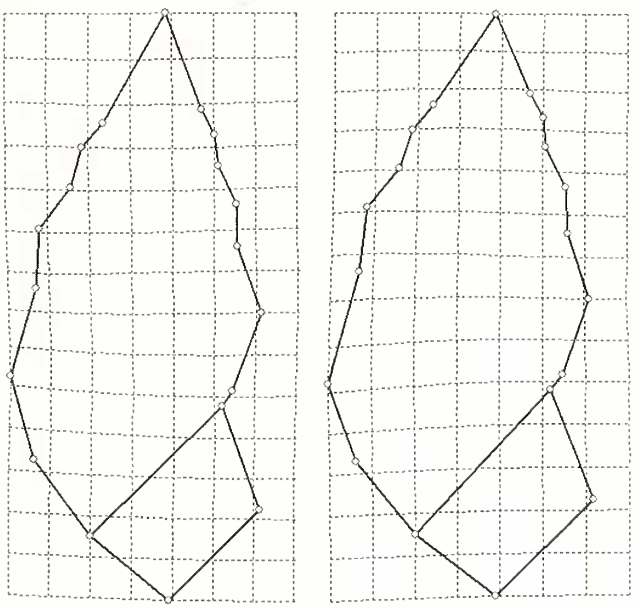


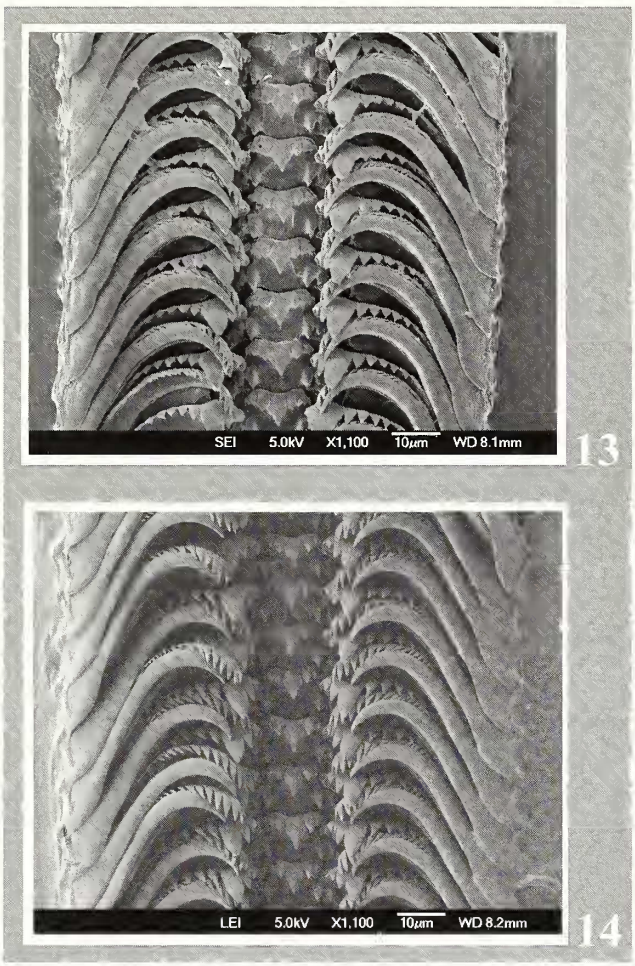
Figure 12. Thin plate spline representations for RW2, showing the deformation of the grid for the average values of *R. guerinii* (left) and *R. lia* (right).

erroneously classified specimens of *R. lia* were observed. In Figure 12 the thin plate spline representation allows interpretation in geometrie terms the positive (eharacteristic of *R. lia*) and negative deviations (characteristic of *R. guerinii*) values for the most signifieant non uniform shape variable, RW2, between the two species.

Radular Analysis: No relevant differenees emerged between the radulae of *R. guerinii* (Figure 13) and *R. lia* (Figure 14) in relation to the shape of teeth and cusps. Rachidian with one median eusp, two pairs of lateral cusps and two pairs of basal cusps. Lateral tooth with a median primary bigger cusp with respectively 2–3 inner cusps and 4–5 outer eusps in *R. guerinii* or 2 inner eusps and 3–5 outer cusps in *R. lia*. Figure 15 represents the frequeney distribution of the number of eusps of the lateral tooth observed in the sample studied and shows higher variability in *R. guerinii* for this charaeter.

Molecular Systematics: The topologies of the P and ML trees (Figure 16 and 17) were comparable: two main clades, corresponding to *R. gueriuii* and *R. lia*, clearly separated from each other and from the outgronps by high bootstrap values.

Two individual sequences of *R. guerinii* formed a weakly supported clade elosely related to the main one of *R. gueriuii*. The MJ network (Figure 18) showed two eonsistently distinct (18 differences) elusters of haplotypes (corresponding to *R. guerinii* and to *R. lia*). *R. gueriuii* displayed higher genetic structure than *R. lia*, with one haplotype occurring in 29 specimens, 2 oecurring in 2 specimens and 11 unique haplotypes. No single haplotype of *R. lia* appeared to be dominant: the most common ones oeeurred in 11, 8, 5, 5 and 2 specimens; 3 were unique. Both clusters were consistently distant from 5 out of 6 outgroups, with *R. lia* closer than *R. guerinii*. Unexpectedly *R. auriscalpium* revealed very little difference (1–2) from some *R. gueriuii* haplotypes.



Figures 13–14. SEM photographs or the radular ribbon of *R. guerinii* (13) and *R. lia* (14). Scale bar = 10 µm.

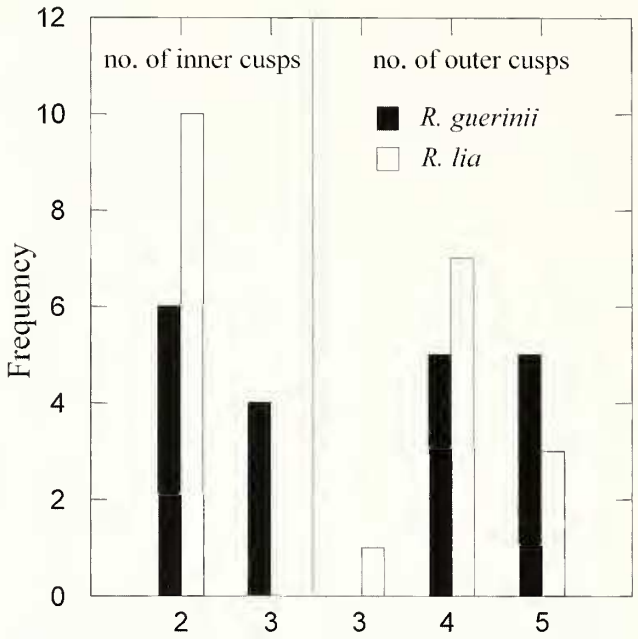


Figure 15. Frequency histograms of distribution of inner and outer cusps in the lateral tooth of *R. guerinii* and *R. lia*.

Dautzenberg Collection Material

Shell Visual Observations: *Rissoa guerinii*: The shells of the lot Rga were eonsiderably worn, younger whorls in most uniformly whitish, in some uniformly yellowish; older 2–4 whorls always pale violet. Most of the shells of Rgb showed worn whitish/yellowish lowermost whorls and remaining whorls with the pigmentation described for *R. guerinii* typical. Shells of Rgc were better preserved and showed the chessboard (or zigzag) pattern deseribed above for *R. guerinii* "var. *conspersa*".

Rissoa lia: Shells of Rlma and Rlmb were topotypes, the former being also from Monterosato collection. Although these shells were rather worn, the two colour varieties, described above for *R. lia*, were still detectable on the younger whorls but had completely disappeared from the whitish oldest whorls.

Shells of Rlp and Rlt were worn but a different pattern, with a whitish spiral band running in the middle of the youngest whorls on a dark brown background.

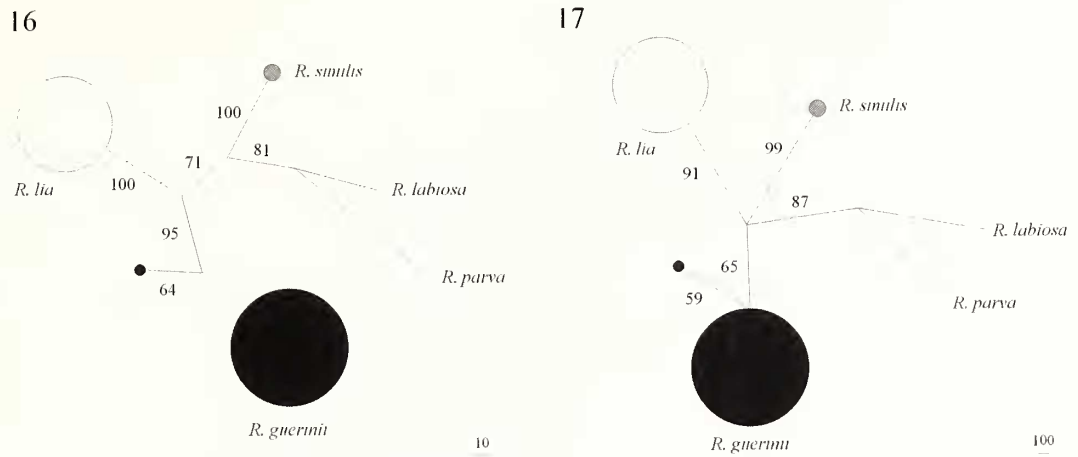
DISCUSSION

Shell and Head-foot: While in the early malacological literature satisfaetory descriptions of the shell of *R. guerinii* are available (Jeffreys, 1869; Fretter and Graham, 1978), deseriptive data on *R. lia* are limited to its essential original description (Monterosato, 1884; page 139).

Verduin (1985) provided a reinterpretation of the two taxa, stressing an extremely close morphologieal resemblanee between them. However he based his diagnosis only on museum dry material, often in a poor state of preservation. As an example, the eharaeter "punetate spiral striae" (Verduin, 1985, pages 112 and 114), reported for the shells of both species, should be considered the result of the deterioration of the subtle reticulate pattern, formed by growth lines and spiral ridges and easily visible in fresh shells.

The redescriptions provided here are based on fresh shells at terminal growth, whieh maintain the peeuliar characters of ornamentation and pigmentation of each species, but at the same time are readily matehed with original muscum material.

Dautzenberg and Durouchoux (1914), on the basc of the material of the lots here named Rga, Rgb, and Rge, deseribed four colour varieties for *R. guerinii*: typical (white with the intervals among ribs brown), *conspersa* (brown background with a chessboard pattern formed by very small spots), *albina* (totally white) and *bipartita* (with the first five or four whorls dark violet and the remaining entirely white). Based on the same material, Verduin (1985) even suggested the presence of a northern *R. guerinii* subspeeies (showing the latter two color



Figures 16–17. Parsimony (16) and Maximum Likelihood (17) Trees drawn after the analysis of 16S sequences of *R. guerinii* and *R. lia*. Circle radius is proportional to sequences frequency. Bootstrap values are given at the left side of each branch.

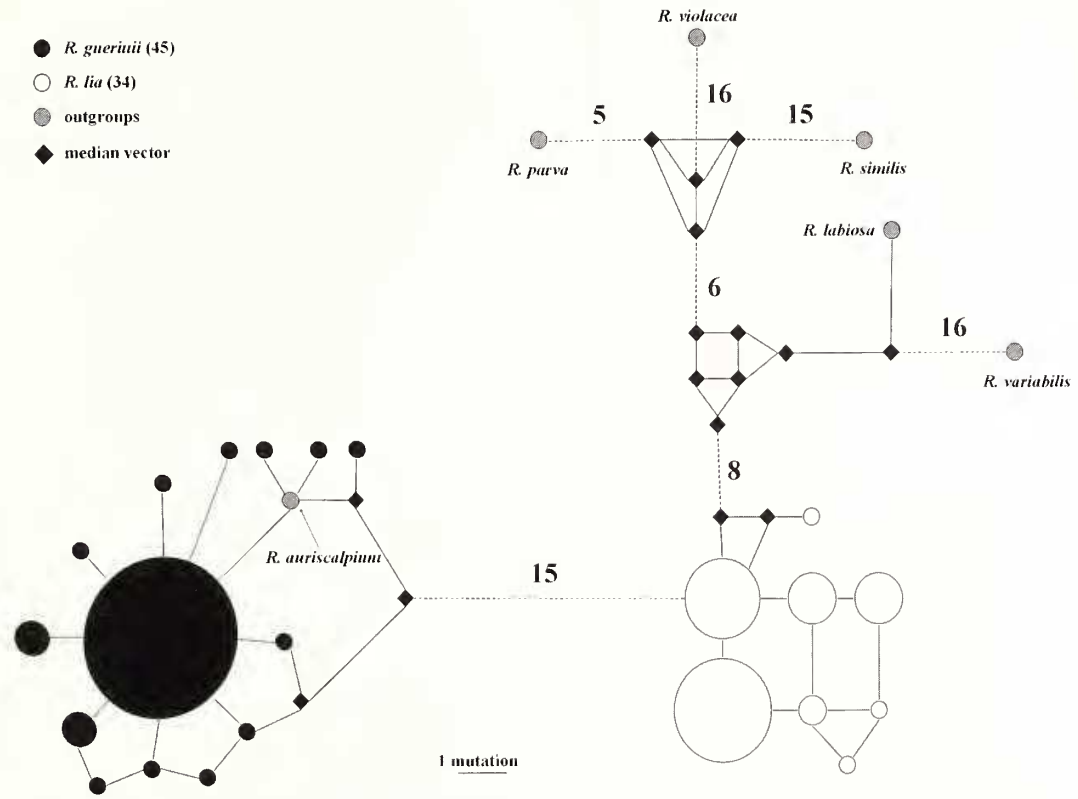


Figure 18. Median Joining Network drawn on the basis of 16S sequences. Number of sequences employed in brackets. Circles radius proportional to haplotype frequency. Dashed lines represent mutational distance higher than 4 (values reported nearby).

varieties) and a southern one (showing the former ones). We cannot see any justification for these claims as the varieties *albina* and *bipartita* are the result of the deterioration of the typical pigmentation, as shown by the worn shells forming the lots Rga and Rgb.

Although his interpretation of *R. lia* (based on the observation of Rlma and Rlmb lots) is generally correct, Verduin (1985: 114) added to its species diagnosis the observation that "in a sample from Trapani, Sicily (fig. 25) many colours and colour patterns can be discerned, among which the colour pattern *conspersa* known in *R. guerinii*."

This observation apparently suggested to Verduin (1985) the idea to move *R. lia* from the subgenus *Apicularia* Monterosato, 1884, to *Goniostoma* Villa, 1884 (the same subgenus of *R. guerinii*). We regard his observation as incorrect and in disagreement with the original description of Monterosato (1884) and with our observations on the material in this study. We found no evidence

that the color pattern *conspersa* belongs to *R. lia*. Although we could not examine the sample mentioned by Verduin (1985), an examination of the figures he provided (25a–h), allowed us to assess that these figures are composed by a mixture of shells of *R. lia* and *R. guerinii* shells, associated because of their similar apex dimensions.

However, the statement that some *R. lia* show "the presence of broken brownish colour lines which encircle the shells" (Verduin, 1985: 114), is in agreement with observations made on lots Rlp and Rlt and with some recent peculiar records of *Rissoa* sp. from Sardinia (Tyrrhenian Sea), showing this same peculiar coloration (Fasulo, pers. comm.). Although this coloration is not typical of the type material of *R. lia*, further observation on live-collected material from these localities is needed to properly address the issue.

Rissoa species tend to maintain the head-foot colour pattern (i.e., position and dimensions of elements as spots or blotches), although they may show intraspecific variation in colour intensity (Fretter and Graham, 1978). This phenomenon has been employed to support species identity (*R. parva* and *R. interrupta*, Warén, 1996; *R. guerinii* and *R. panhormensis*, Criscione et al., 2009) or to hypothesize recent speciation (*R. membranacea* type A and B, Rehfeldt, 1968; *R. auriscalpium* type a and b, Colognola et al., 1986). This rule is in agreement with the slight variation in color intensity observed in this study for the two varieties of *R. guerinii*, but it does not apply to *R. lia*, whose color varieties showed two distinct patterns.

Traditional Morphometry: Although the present morphometric analysis is restricted to populations from a single locality, its results are not only helpful in understanding intrapopulation variability in shell morphology, but also in drawing more general conclusions in terms of specific differentiation. The differences observed can be expressed in terms of teleoconch and protoconch variation, with the first element being by far the most important. Populations of both species show a wide variability in protoconch dimensions, which is larger for *R. guerinii*. *Rissoa lia* shows twice the intrapopulation variation in teleoconch characters than that observed for *R. guerinii*.

The values of shell parameters obtained for the two species showed a considerable overlap, which is mainly dependent on the strong similarity in protoconch dimensions and not, surprisingly, by the resemblance of the teleoconchs. Most shells of *R. guerinii* appear to have a larger relative size, a more elongated aperture and to be more slender than most *R. lia* shells.

Discriminant analysis showed that *R. guerinii* and *R. lia* populations can be distinguished mainly for the more elongated aperture of *R. guerinii* shells, for their larger relative size, and the higher slenderness. The importance of protoconch is only marginal.

In summary, our results show that teleoconch morphometry can be efficiently used to discriminate between *R. guerinii* and *R. lia*, whereas protoconch dimensions are less representative of the overall interspecific variation and less reliable, due to the large overlap shown. This idea is in contrast with the view of the discriminating power of protoconch dimensions (Verduin, 1986).

Some Methodological Considerations: Cadèe (1998: 91) critically revised the methodology used by Verduin (1976, 1982b, 1985, 1986) highlighting that the measurements of d and D_0 "depend on the accurate vertical position of the shell and the somewhat arbitrary location of the line along which d and D_0 are measured." Cadèe (1998) dealt with the former issue by performing repeated measurements of d and D_0 on specimens of *R. membranacea*, whose vertical placement was reiterated ten times, and obtained a standard deviation of about 0.01 mm for both the variables. This value is roughly the same as that which separates the mean values of d and D_0 of *R. lia* and *R. guerinii* in this study (not shown). This means that some of the differences in protoconch dimensions might be indeed the consequence of the inaccurate measurements, rather than representing real variation. The alternative solution of performing measurements on digital photographs has been also recently employed (Criscione et al., 2009). The second issue is that real *Rissoa* protoconchs often do not correspond to the ideal shell apex figured by Verduin (1977; Figure 1), but most of them are rather irregular. In protoconchs like these there is a large range of possibilities to locate the line along which d and D_0 should be measured and the choice is largely arbitrary.

Geometric Morphometry: The ANOVA revealed a significant larger size (CS) of the shells of *R. guerinii* compared to those of R. *lia*, confirming the results of visual observations and traditional morphometry. ANOVA showed a significant difference ($p<0.05$) between the two groups in the first (RW1) and the second (RW2) non-uniform shape variables. The significance for RW2 remained unaltered when correcting the analysis for CS (ANCOVA), while that of RW1 was not significant, indicating that the shape difference explained by that variable was dependent on size. This means that the two groups differ exclusively on the second non-uniform shape variable (RW2), independently from the correlation between shape and size.

The discriminant function calculated from all the non-uniform shape variables, was successful in morphometrically discriminating the two groups. RW2 was the most important variable in determining the distinction between *R. guerinii* and *R. lia*. The mean values of this variable for each of the two groups (positive for *R. lia* and negative for *R. guerinii*) were plotted in a tps representation (Figure 12). The plot showed that that variable RW2 is a reflection of the most obvious discernable shell shape difference. This comprised the more fusiform shape of R. *guerinii*, contributed by consistently narrower whorls than those of *R. lia* (represented by the contraction of the corresponding zone of the grid) and a substantially equal penultimate whorl.

The grid deformation in the plot of *R. lia* increase progressively from the top to the middle-lower as expeeted for a more eonical shape. In the same plot, larger deformations of the upper part and the lower of the grid for *R. lia*, account respectively for the more obtuse apex and the larger aperture of *R. lia*.

Radular Analyses: Although the taxonomical value of radular morphology in rissoids is exclusively limited to generic level (Ponder, 1985), eomparative analysis of patterns of similarity may be helpful in resolving the complexity of a highly diverse genus such as *Rissoa*. Along with other charaeters, differences in the size of the cusps of marginal tooth have been employed to show differenees between two morphs of *R. auriscalpium* (Colognola et al., 1986) and radular identity has been used to support findings of recent speciation in *R. membranacea* morphs (Rehfeldt, 1968).

However, detailed reports on the taxonomie value of eusps in rissoids are laeking and this makes it difficult to give the appropriate weight to the difference between *R. guerinii* and *R. lia* in the relative number of cusps of the lateral tooth. But the pattern emerged appears to be rather eonstant and its eventual value as distinctive character of phylogenctic significance cannot be excluded.

Molecular Systematics: Analysis of mitochondrial sequenees of gastropod taxa, involved in a recent speciation event (or an ongoing speciation process), often show traces of introgression (e.g., Kirby et al., 1997; Kojima et al., 2001). Introgression shown by 16S sequences has been used to support ongoing speciation in the P/NP pair of sympatrie siblings *Rissoa auriscalpium/R. italiensis* (Panieo and Patti, 2005). In this study, no evidenee of introgression was detected for sympatric populations of *R. guerinii* and *R. lia*, suggesting that claims of recent speciation are not acceptable. However, the evolutionary rate of 16S rRNA is not known in rissoids and the hypothesis of an earlier cladogenetie event, giving rise to *R. guerinii* and *R. lia*, cannot be exeluded. The results of our moleeular analysis also challenge the likelihood of this scenario.

The ML analysis (Figure 17) failed in resolving the polytomy resulted for sequences of *R. guerinii*, *R. lia* and one of the outgroups, *R. similis*, as expected if our ingroups were sister species. Before Verduin (1985) moved *R. lia* to the subgenus *Goniostoma*, a closer relationship between this species and *R. similis* was commonly aecepted (as both members of the subgenus *Apicularia*). Our results may be considered in agreement with this idea.

The hypothesis of the shared origin of *R. guerinii* and *R. lia* would require much lower mutational distance between the two sister speeies than between each sister speeies and the outgroups. Our MJ network (Figure 18) shows that this is not the ease. *R. lia* is separated from the main haplotype of *R. guerinii* by a distance of the same order of magnitude than the distance separating this species from the other outgroups.

The significant difference in haplotype diversity of *R. guerinii* and *R. lia* (shown by the same analysis) may be the result of stochastie events in the evolutionary history of these lineages or may be alternatively related to the supposed alternative strategy of larval dispersal (P/NP). *Rissoa guerinii* displays a higher genetic structure which may reflect a higher genetic flow, due to its higher dispersal capability. Conversely, *R. lia* shows a lower strueture which would reflect a lower level of gene flow. Despite records of *R. guerinii* planktotrophie veligers have been long reported (Lebour, 1934; Thiriot-Quievreux and Babio, 1975), no evidence for the non-planktotrophic strategy of *R. lia* are known and sueeessful laboratory experiments on spawning are lacking. The actual strategy of dispersal of the two species is thus still to be confirmed.

Surprisingly, an outgroup sequence of *R. auriscalpium* showed only two differences from the main *R. guerinii* haplotype. This short distance fits in the normal intraspecifie (and even intrapopulational) variability of *R. guerinii* and represent a clue of previously unsuspected genetic similarity between the two speeies. Further investigation is required to properly address the issue.

CONCLUSIONS

The taxonomic revisions of Verduin (1976, 1977, 1982b, 1985, 1986), although based exclusively on empty shells, have represented landmarks in the α-taxonomy of *Rissoa*. Despite some evidence of the taxonomic unreliability of shell characters in *Rissoa* (*e.g.*Wigham, 1975; Warén, 1996), only a few attempts have been made to dispute his conclusions. These include eriticism of his methods (Cadèe, 1998) and the utilization of non shell-based approaehes (Colognola *et al.*, 1986; Panico and Patti, 2005; Criscione et al., 2009). This trend has been followed in our work, which demonstrates the value of a synergy between modern techniques applied to the analysis of shell charaeters and molecular methods. The present study has shown that the very similar shell morphology of *R. guerinii* and *R. lia* hides a clear distinction in other characters, indicating that they should not be considered as a eryptic pair of sister taxa.

ACKNOWLEDGMENTS

We are grateful to Dr. Danilo Scuderi (Università di Catania) for having provided the drawings shown in Figures 6–9. We would also like to thank Prof. Jackie Van Goethem and Prof. Thierry Backeljau (RBINS) for the possibility to study Dautzenberg collection material. We want to express gratitude to Prof. Domenieo Caruso and Prof. Grazia Cantone (Università di Catania) for financial support, the Molecular Biology Service and the Scanning Electron Mieroseopy Service of Stazione Zoologica "Anton Dohrn" (Naples). We are also thankful to Dr. Winston F. Ponder and an anonymous referec for their valuable comments.

LITERATURE CITED

Bandelt, H.J., P. Forster, and A. Rohl. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16: 37–48.

Bouchet, P. 1989. A review of poecilogony in gastropods. Journal of Molluscan Studies 55: 67–78.

Cadèe, G.C. 1998. *Rissoa membranacea* (J. Adams, 1800) (Gastropoda, Prosobranchia) from the Dutch Wadden Sea. Basteria 61: 89–98.

Colognola, R., P. Masturzo, G.F. Russo, M. Scardi, D. Vinci, and E. Fresi. 1986. Biometric and genetic analysis of the marine rissoid *Rissoa auriscalpium* and its ecological implications. Marine Ecology, 7: 265–285.

Criscione, F., Scuderi, D., and F.P. Patti. 2009. Revising α-taxonomy in shelled gastropods: the case of *Rissoa panhormensis* Verduin, 1985. The Nautilus 123: 303–312.

Fretter, V. and A. Graham. 1978. The prosobranch molluscs of Britain and Denmark. Part 4. Marine Rissoacea. Journal of Molluscan Studies Supplement 6: 151–241.

Jablonski, D. and R.A. Lutz. 1983. Larval ecology of marine benthic invertebrates - paleobiological implications. Biological Reviews of the Cambridge Philosophical Society 58: 21–89.

Jeffreys, J.G. 1867. British Conchology. Volume IV. Van Voorst, London. 486 pp.

=Knowlton, N. 1986. Cryptic and sibling species among the decapod Crustacea. Journal of Crustacean Biology 6: 356–363.

Kojima, S., N. Ota, K. Mori, K., T. Kurozumi, and T. Furota. 2001. Molecular phylogeny of Japanese gastropods in the genus *Batillaria*. Journal of Molluscan Studies 67: 377–384.

Lebour, M.V. 1934. Rissoid larvae as food of the young herring. The eggs and larvae of the Plymouth rissoides. Journal of Marine Biological Association of United Kingdom 19: 523–539.

Monterosato, T.A. d. 1883–1885. Conchiglie littorali mediterranee. Naturalista Siciliano 3(3): 87–91 (1883); 3(4): 102–111; 3(5): 137–140; 3(6): 159–163; 3(8): 227–231; 3(10): 277–281; 4(1–2): 21–25; 4(3): 60–63 (1884); 4(4): 80–84; 4(8): 200–204.

Oliverio, M. 1996. Contrasting developmental strategies and speciation on north-east Atlantic prosobranchs: a preliminary analysis. In: Taylor, J.D. (ed.) Origin and evolutionary radiation of the Mollusca. Oxford Science Publications, Oxford, pp. 261–266.

Panico, M. and F.P. Patti. 2005. Poecilogenesis in *Rissoa auriscalpium* complex (Coenogastropoda: Rissoidae). Poecilogony engine of speciation. Congresso Internazionale delle Società Europee di Malacologia, Naples, October 2005. Poster presentation.

Ponder, W. 1985. A review of the genera of the Rissoidae (Mollusca: Mesogastropoda: Rissoacea). Records of the Australian Museum 4 (Suppl.): 1–221.

Posada, D. and K.A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Récluz, C.A. 1843. Catalogue descriptif de plusieurs nouvelles espèces de coquilles de France suivi d'observations sur quelques autres. Revue zoologique, par la Société Cuvierienne. 6: 5–12, 104–112, 228–238, 257–261.

Rehfeldt, N. 1968. Reproductive and morphological variations in the prosobranch Rissoa membranacea. Ophelia 5: 157–173.

Rohlf, J.F. 2007a. TpsDig2 version 2.10. Available via http://life.bio.sunysb.edu/morph/soft-tps.html (Accessed July 2008).

Rohlf, J.F. 2007b. TpsRelw version 1.45. Available via http://life.bio.sunysb.edu/morph/soft-tps.html [Accessed July 2008)].

Strathmann, R.R. 1978a. Evolution and loss of feeding larval stages of marine invertebrates. Evolution 32: 894–906.

Strathmann, R.R. 1978b. Progressive vacating of adaptive types during the Phanerozoic. Evolution 32: 907–914.

Swofford, D.L. 2003. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b 10. Sinauer Associates, Sunderland.

Thiriot-Quiévreux, C. and C.R. Babio. 1975. Etude des protoconques de quelques prosobranches de la région de Roscoff. Cahiers de Biologie Marine 16: 135–148.

Thorson, G. 1950. Reproductive and larval ecology of marine bottom invertebrates. Biological Reviews 25: 1–45.

Verduin, A. 1976. On the systematic of recent *Rissoa* of the subgenus *Turboella* Gray, 1847, from the Mediterranean and European Atlantic coasts. Basteria 40: 21–73.

Verduin, A. 1977. On a remarkable dimorphism of the apices in many groups of sympatric, closely related marine gastropod species. Basteria 41: 91–95.

Verduin, A. 1982. How complete are diagnoses of coiled shell of regular build? A matematical approach. Basteria 45: 127–142.

Verduin, A. 1982b. On the taxonomy and variability of Recent European and North African marine species of the subgenus *Rissostomia* Sars, 1878, of the genus *Rissoa* Desmarest, 1814 (Mollusca, Gastropoda, Prosobranchia). Basteria 45: 143–166.

Verduin, A. 1985. On the taxonomy and variability of Recent European and North African marine species of the subgenera *Apicularia* and *Goniostoma* of the genus *Rissoa* (Gastropoda, Prosobranchia). Basteria 49: 105–132.

Verduin, A. 1986. On the systematics of some Recent *Rissoa* (Gastropoda, Prosobranchia). Basteria 50: 13–21.

Warén, A. 1996. Ecology and systematics of the north European species of *Rissoa* and *Pusillina* (Prosobranchia: Rissoidae). Journal of the Marine Biological Association of United Kingdom 76: 1013–1059.

Wigham, G.D. 1975. Environmental influences upon the expression of shell form in *Rissoa parva* (da Costa) [Gastropoda: Prosobranchia]. Journal of the Marine Biological Association of United Kingdom 55: 425–438.